Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US04/038179

International filing date: 15 November 2004 (15.11.2004)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/519,493
Filing date: 13 November 2003 (13.11.2003)

Date of receipt at the International Bureau: 20 December 2004 (20.12.2004)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

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APPLICATION NUMBER: *60/519,493*
FILING DATE: *November 13, 2003*
RELATED PCT APPLICATION NUMBER: *PCT/US04/38179*

Certified by

Jon W Dudas

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PTO/SB/16 (10-01)
Approved for use through10/31/2002. OMB 0651-0032
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No.	EV 022277630 US

INVENTOR(S)

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TITLE OF THE INVENTION (500 characters max)

MIXED COMPLEMENTARY VIRAL VECTORS FOR GENE THERAPY

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 51
☐ Drawing(s) *Number of Sheets*
☑ Application Data Sheet. See 37 CFR 1.76
☐ CD(s), Number
☑ Other (specify) Post Card

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☑ Applicant claims small entity status. See 37 CFR 1.27.
☑ A check or money order is enclosed to cover the filing fees
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FILING FEE AMOUNT ($): $80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☑ No.
☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE _____

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Date 11/13/2003

REGISTRATION NO. *(if appropriate)* 48,921

Docket Number: 13087-106

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

Express Mail Label No: EV 022277630 US
13087-106
Date of Deposit: November 13, 2003
Provisional patent application

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

A PROVISIONAL PATENT APPLICATION

FOR

MIXED COMPLEMENTARY VIRAL VECTORS FOR GENE THERAPY

FIELD OF THE INVENTION

The present invention relates generally to the field of viral vectors and their use in treating disease. Specifically, the instant invention relates to replication-conditional (RC) vectors mixed with replication deficient (RD) vectors and methods for using them.

BACKGROUND OF THE INVENTION

Progress in the study of genetics and cellular biology over the past three decades has greatly enhanced our ability to describe the molecular basis of many human diseases.[4,5] Molecular genetic techniques have been particularly effective. These techniques have allowed for the isolation of genes associated with common inherited diseases that result from a lesion in a single gene such as ornithine transcarbamylase (OTC) deficiency, cystic fibrosis, hemophilias, immmunodeficiency syndromes, and others as well as those that contribute to more complex diseases such as cancer [6,7]. Therefore, gene therapy, defined herein as the introduction of genetic material into a cell in order to either change its phenotype or genotype, has been intensely investigated over the last fourteen years. [5,8]

For effective gene therapy, it is necessary to deliver therapeutic genes to relevant cells *in vivo* at high efficiency, to express the therapeutic genes for prolonged periods of time, and to ensure that the transduction events do not have deleterious effects. To accomplish these criteria, a variety of vector systems have been evaluated. These systems include viral vectors such as retroviruses (including lentiviruses), adenoviruses, adeno-associated viruses, and herpes simplex viruses, and non-viral

systems such as liposomes, molecular conjugates, and other particulate vectors. [5,8] Although viral systems have been efficient in laboratory studies, none have yet been definitevely curative in clinical applications.

Adenoviral and retroviral vectors have been the most broadly used and analyzed of the current viral vector systems, although significant advances have been accomplished in the adeno-associated field. These vectors have been successfully used to efficiently introduce and express foreign genes *in vitro* and *in vivo*. These vectors have also been powerful tools for the study of cellular physiology, gene and protein regulation, and for genetic therapy of human diseases. Indeed, they are currently being evaluated in Phase I, II and III clinical trials. [9,10] However, viral vector systems have significant limitations in delivery and efficacy.

Gene therapy and gene delivery vehicles

Gene therapy vectors can be classified into one of two main types - viral and non-viral. (Both types are reviewed in detail in Methods in Human Gene Therapy, T. Freidmann Ed. Cold Spring Harbor Press, 1999, which is hereby incorporated by reference.) The most commonly used viral systems are retroviral vectors and adenoviral vectors, in part for historical reasons and in part because they have been relatively straightforward to make in clinically useful quantities. These vectors have both been used extensively in the clinic, and some clinical trials have also been conducted using adeno-associated viral vectors, rhabdoviruses, herpes viral vectors and vectors based on vaccinia virus or poxviruses. These viruses have various strengths and weaknesses, but are all relatively efficient in delivering genes to target tissues. Limitations include difficulties in making sufficient quantities for some vectors, inability to accurately target the gene delivery *in vivo*, and toxic or immunological side effects of viral gene products. However, it should be noted that even with a relatively efficient viral vector, it is not reasonable at present to expect that a gene can be delivered to every sick cell, and so therapy needs to be accomplished by means that are compatible with this issue.

Non viral systems include naked DNA, DNA formulated in lipososomes, DNA formulated with polycation condensing agents or hybrid systems and DNA conjugated with peptides or proteins, such as single chain antibodies, in order to target them to specific tissues. These systems are more amenable to constructing rationally regulated steps in order to accomplish a long *in vivo* half-life, delivery to the target cell/tissue, entry into the cytoplasm and nucleus, and then subsequent expression. Although there are possible solutions to each of these issues, they have not yet been efficiently combined, and efficiency of gene transfer *in vivo* remains an issue at this time. So for these systems also, it is not reasonable to expect to be able to deliver a gene to every cell, for example in a tumor.

Therefore, in gene therapy, *e.g.* cancer therapies, using gene delivery vehicles, it is necessary to use mechanisms that allow some kind of amplification of the gene delivery event. These can include stimulation of the immune system, various forms of bystander effects, spread of apoptosis, antiangiogenic effects, pro-coagulant effects, replication competent viral vectors or other mechanisms.

Adenoviral Vectors

Adenoviridae is a family of DNA viruses first isolated in 1953 from tonsils and adenoidal tissue of children. [11] Six sub-genera (A, B, C, D, E, and F) and more than 49 serotypes of adenoviruses have been identified as infectious agents in humans. [12] Although a few isolates have been associated with tumors in animals, none have been associated with tumors in humans. The adenoviral vectors most often used for gene therapy belong to the subgenus C, serotypes 2 or 5 (Ad2 or Ad5). However, other human and non-human adenoviruses and chimeric adenoviruses can also be used. Infection by Ad2 or Ad5 results in acute mucous-membrane infection of the upper respiratory tract, eyes, lymphoid tissue, and mild symptoms similar to those of the common cold. Exposure to type C adenoviruses is widespread in the population with the majority of adults being seropositive for this type of adenovirus[12].

Adenovirus virions are icosahedrons of 65 to 80 nm in diameter containing 13% DNA and 87% protein. [13] The viral DNA is approximately 36 kb in length and is naturally found in the nucleus of infected cells as a circular episome held together by

the interaction of proteins covalently linked to each of the 5' ends of the linear genome. The ability to work with functional circular clones of the adenoviral genome greatly facilitated molecular manipulations and allowed the production of replication defective vectors.

Two aspects of adenoviral biology have been critical in the production of commonly used adenoviral vectors. First is the ability to have essential regulatory proteins produced in *trans,* and second is the inability of adenovirus cores to package more than 105% of the total genome size. [14] The first was originally exploited by the generation of 293-cells, a transformed human embryonic kidney cell line with stably integrated adenoviral sequences from the left-hand end (0-11 map units) comprising the E1 region of the viral genome. [15] These cells provide the E1A gene product in *trans* and thus permit production of virions with genomes lacking E1A. Such virions are considered replication deficient (RD) since they can not maintain active replication in cells lacking the E1A genes (although replication may occur at high vector concentrations). The 293 cells are permissive for the production of these replication deficient vectors and have been utilized in all approved protocols that use adenoviral vectors.

The second was exploited by creating backbones that exceed the 105% limit to force recombination with shuttle plasmids carrying the desired transgene. [16] Most currently used adenoviral vector systems are based on backbones of subgroup C adenovirus, serotypes 2 or 5. [14] Deleting regions E1/E3 alone or in combination with E2/E4 produced first- or second-generation replication-defective adenoviral vectors, respectively. [14] As mentioned above, the adenovirus virion can package up to 105% of the wild-type genome, allowing for the insertion of approximately 1.8 kb of additional heterologous DNA. The deletion of E1 sequences adds another 3.2 kb, while deletion of the E3 region provides an additional 3.1 kb of foreign DNA space. Therefore, E1 and E3 deleted adenoviral vectors provide a total capacity of approximately 8.1 kb of heterologous DNA sequence packaging space. Deletion of E2 and E4 regions further increase the available space of the vectors and increase the safety of the multiplely deleted backbones. Multiplely deleted backbones, including the completely deleted "helper-dependent" backbones tend to have increased duration

of transgene expression. The principal benefit of RD vectors is their capacity to carry large segments of heterologous DNA that can convey therapeutic benefits, and, since they do not replicate, expression lasts longer then the normal life cycle of a replication competent lytic virus. The major hurdle is delivery of well distributed virus load to the tissue of interest.

Adenoviruses have been extensively characterized and make attractive vectors for gene therapy because of their relatively benign symptoms even as wild type infections, their ease of manipulation *in vitro*, the ability to consistently produce high titer purified virus, their ability to transduce quiescent cells, and their broad range of target tissues. In addition, adenoviral DNA is not incorporated into host cell chromosomes minimizing concerns about insertional mutagenesis or potential germ line effects. This has made them very attractive vectors for tumor gene therapy protocols and other protocols in which transient expression may be desirable. However, these vectors are generally not very useful for metabolic diseases and other application for which long-term expression may be desired. Human subgroup C adenoviral vectors lacking all or part of E1A and E1B regions have been evaluated in Phase I clinical trials that target cancer, cystic fibrosis, and other diseases without major toxicities being described. [8, 9, 17, 18] A major exemption to the safety of these vectors was the death of a young man that received a very large dose of E1, E4 deleted vector directly into the hepatic artery. The large bolus dose of adenoviral virions led to liver toxicity, a DIC-like response and ultimately respiratory distress and death.

The use of "replication conditional" (RC) adenoviruses for cancer therapy has shown some effects in clinical studies. "Replication conditional" are vectors or viruses that either lack a portion of the genome which is important for replication in "normal" cells, but less critical in the target cells (*e.g.*, Onyx 015, which is a natural mutant missing p53 responsive E1B functions), or contain regulatory elements that target specific tissues (*e.g.*, a tissue or condition specific promoter/enhancer for the expression of the E1A, E1B, E2, or E4 regions of the virus). The major benefit of these RC vectors is their potential for *in vivo* amplification. A major concern for the

efficacy of RC vectors is the limited ability to carry heterologous genes and the self limiting duration of expression due to their cytolytic effect.

Thus, RD and RC vectors have complementary strengths and weakness that can be overcome by mixing the two vector types together. To address the issue of limited distribution from RD vectors, they can be delivered with RC vectors for *in vivo* amplification in the target tissue. To address the issue of limited space and shortened duration of trangene expression from the RC vectors, the RD vector can carry the therapeutic genes. The RC vector can also carry therapeutic genes but these can not express as long as the non-replicating constructs.

The RC and RD combination is not limited to adenoviral vectors. For example, the *trans*-complementing functions for a RD adenoviral vector may be carried in a RC herpes vector.

SUMMARY OF THE INVENTION

The present invention relates generally to the field of viral vectors and their use in treating disease. Specifically, the instant invention relates to replication-conditional (RC) vectors mixed with replication deficient (RD) vectors and methods for using them.

In one embodiment of the present invention, a viral vector comprising a nucleic acid sequence sufficient for *in vivo* viral production is disclosed. In one aspect, the vector comprises a first nucleotide sequence encoding sufficient sequence for the production of replication competent and replication deficient viruses. In one aspect, this viral vector is delivered to a target site in a viral form. In another aspect, this viral vector is delivered in a non-viral form. This delivery of the vector can be directed to specific target tissues by the addition of regulatory nucleotide sequences or mutations of the natural regulatory sequences or combinations of the above to limit production of viral vectors within the specific tissues or conditions of tissues, such as tumors. In a further aspect, the nucleotide sequence of this vector can be directed to specific target tissues by the addition of conjugated molecules, such as polycations, peptides, antibodies, single chain antibodies or combinations of the above. In another aspect, the viral vector comprises a second nucleotide sequence that encompasses a

sequence sufficient for being packaged within a viral capsid produced from the first nucleotide sequence and in addition contains nucleotide sequences encoding one or more therapeutic genes.

In another embodiment of the present invention, a vector comprising substantially all of the adenoviral genome is disclosed. In this embodiment, the regulatory elements of the adenoviral genome, such as the E1 genes, are under the regulatory control of the host cell's associated sequences. In one aspect, post-transcriptional or post-translational host cell effects, such as the permissivity for intron excission or complex enzyme formation, mediate the control of the vector's nucleotide sequence expresson.

In another embodiment of the present invention there is a nucleic acid sequence as described above and a nucleic acid region for targeting an adenoviral vector.

In another embodiment of the present invention, genetic complementation occurs between an exogenous nucleic acid and a host cell's genome, wherein an intervening exogenous nucleotide sequence replaces an endogenous nucleotide sequence of the host cell's genome, thereby forming a functional self-splicing intron. In one aspect, the host cell is an hepatocyte. In another aspect, the endogenous nucleotide sequence is selected from the group consisting of a reporter region, *ras*, *myc, raf, erb, src, fms, jun, trk, ret, gsp, hst, bcl abl,* Rb, CFTR, p16, p21, p27, p53, p57, p73, C-CAM, APC, CTS-1, zac1, scFV *ras*, DCC, NF-1, NF-2, WT-1, MEN-I, MEN-II, BRCA1, VHL, MMAC1, FCC, MCC, BRCA2, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11 IL-12, IL15, IL18, GM-CSF, G-CSF, TNF, gIFN, aIFN, bIFN, thymidine kinase, cytosine deaminase, cyt-p450, CD40L, Factor VIII, Factor IX, CD40, multiple disease resistance (MDR), ornithine transcarbamylase (OTC), ICAM-1, HER2-neu, PSA, terminal transferase, caspase, NOS, VEGF, endostatin, vegostatin, FGF, FGF4, bFGF, HIS, heat shock proteins, IFN α and γ, TNF α and β, telomerase, and insulin receptor.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates generally to the field of viral vectors and their use in treating disease. Specifically, the instant invention relates to replication-conditional (RC) vectors mixed with replication deficient (RD) vectors and methods for their use.

RC vectors are able to selectively replicate in a target tissue in order to provide a therapeutic benefit from the presence of the vector *per se* or from heterologous gene products expressed from the vector and distributed throughout the tissue. In RC vectors, replication is conditioned on the presence or absence of a factor(s) that induces or inhibits transcription or function of the gene by means of a regulatory sequence. With these RC vectors, therefore, a target tissue can be selectively treated.

RD vectors are such that they lack essential sequences for their replication. RD vectors can only be amplified in cells in which these missing functions are provided in *trans*. When provided in *trans*, the functions complement the RD vectors so they can replicate and be amplified. Such vectors are relatively safe for *in vivo* use and have the capacity to carry heterologous sequences within their genome. The heterologous sequences can contain sequences, that when expressed, convey therapeutic functions, such as immunostimulation (*e.g.* cytokines, chemokines, superantigens and others), cytotoxicity (*e.g.* viral thymidine kinase, cytosine deaminase, cytochrome p450 and others), cell-cycle control (*e.g.* p53, RB, and others) or the heterologous sequences may complement a function of the first viral vector.

The invention relates to compositions and methods of using RC and RD vectors in combination. More specifically, the invention relates to using RC vectors to amplify RD vectors *in vivo*.

The term "adenoviral" as used herein is defined as associated with an adenovirus.

The term "adenoviral inverted terminal repeat flanking sequences" as used herein is defined as a nucleic acid region naturally located at both of the 5' and 3' ends of an adenovirus genome which is necessary for viral replication.

The term "adenovirus" as used herein is defined as a DNA virus of the Adenoviridae family.

The term "cassette" as used herein is defined as a nucleic acid which can express a protein, polypeptide or RNA of interest. In one aspect, the nucleic acid is positionally and/or sequentially oriented (*i.e.*, operably linked) with other necessary elements so it can be transcribed and, when necessary, translated. In another aspect, a protein, polypeptide or RNA of interest is for therapeutic purposes, such as the treatment of disease or a medical condition.

The term "E4" as used herein is defined as the nucleic acid region from an adenovirus and encodes numerous polypeptides known in the art, including a polypeptide which binds to the nuclear matrix and another polypeptide which is associated with a complex including E1B (e.g. Genbank Accession No. BK000408, "Human adenovirus type 5, complete genome", E1 region- nt.560-3509; E4 region- nt. 32914-35526).

The term "flanking" as used herein is referred to as being on either side of a particular nucleic acid region or element.

The term "internal region" as used herein is defined as the nucleic acid region that is present within viral flanking sequences. The internal region can also include regulatory elements and exogenous sequences such as a transactivator and/or a suicide nucleic acid region.

The term "nucleic acid (or nucleotide) of interest" as used herein is defined as a nucleic acid that is utilized for therapeutic purposes or for control of viral replication for gene therapy in the vectors of the present invention. In one aspect, a nucleic acid sequence of interest is a gene or a portion of a gene. In another aspect, a nucleic acid of interest is a viral regulatory gene. In another aspect, a nucleic acid sequence of interest is a gene or a portion of a gene. In yet another aspect, a nucleic acid sequence

is a promoter/enhancer region controlling the expression of a gene. In still another aspect, a nucleic acid of interest is an adenoviral E1, E4 or E2 gene (e.g. Genbank Accession No. BK000408, "Human adenovirus type 5, complete genome", E1 region- nt.560-3509; E4 region- nt. 32914-35526; E2 region- 35606- 4057).

The term "suicide nucleic acid region" as used herein is defined as a nucleic acid which, upon administration of a prodrug, effects transition of a gene product to a compound which kills its host cell. Examples of suicide gene-prodrug combinations which can be used are Herpes Simplex Virus-thymidine kinase (HSV-tk) [the gene] and ganciclovir, acyclovir, valacyclovir, valgancyclovir, penciclovir or FIAU; oxidoreductase and cycloheximide; cytosine deaminase and 5-fluorocytosine; thymidine kinase thymidilate kinase (Tdk::Tmk) and AZT; and deoxycytidine kinase and cytosine arabinoside [the prodrug].

The term "therapeutic nucleic acid" as used herein is defined as a nucleic acid region, which can be a gene, that provides a therapeutic effect on a disease, medical condition, or characteristic (including genotype and phenotype) to be modified within an organism.

The term "transactivator" as used herein is defined as a biological entity such as a protein, polypeptide, oligopeptide or nucleic acid that regulates expression of a nucleic acid. In one aspect, the transactivator is the *tet* transactivator.

The term "vector" as used herein is defined as a vehicle comprising a nucleic acid for the delivery of the one or more predetermined nucleotide sequences into a host cell. The vector can be a linear molecule, a circular molecule, or a virion.

Plasmid vectors of the present invention do not necessarily increase the risks presently associated with the described viral vectors. The plasmids described herein can be used in the laboratory for vector production or can be used *in vivo* for vector delivery. These allow for the exploitation of ease of plasmid production, the lack of plasmid immunogenicity, the potential for plasmid targeted delivery, and the ability of *in vivo* vector amplification. They also provide unique advantages, for example, non-immunogenic *in vivo* delivery to facilitate systemic delivery.

The term "adenovirus" as used herein is defined as a DNA virus of the Adenoviridae family. A skilled artisan is aware that a multitude of human adenovirus (mastadenovirus H) immunotypes exists including Type 1 through 42 (including 7a), non-human adenoviruses from simians, canines, bovines and other hosts have also been characterized and sequenced. The description of Adenoviridae including genome organization and replication, is detailed in references known in the art, such as Fields Virology (Fields *et al.*, eds.).

In one embodiment of the present invention, a viral vector comprising a nucleic acid sequence sufficient for *in vivo* viral production is disclosed. In one aspect, the vector comprises a first nucleotide sequence encoding sufficient sequence for the production of replication competent and replication deficient viruses. In one aspect, this viral vector is delivered to a target site in a viral form. In another aspect, this viral vector is delivered in a non-viral form. This delivery of the vector can be directed to specific target tissues by the addition of regulatory nucleotide sequences or mutations of the natural regulatory sequences or combinations of the above to limit production of viral vectors within the specific tissues or conditions of tissues, such as tumors. In a further aspect, the nucleotide sequence of this vector can be directed to specific target tissues by the addition of conjugated molecules, such as polycations, peptides, antibodies, single chain antibodies or combinations of the above. In another aspect, the viral vector comprises a second nucleotide sequence that encompasses a sequence sufficient for being packaged within a viral capsid produced from the first sequence and in addition contains a nucleotide sequence encoding a therapeutic gene.

In one aspect of the present embodiment, the nucleotide sequence for *in vivo* delivery is comprised of one or more nucleic acid sequences necessary for other replication competent or conditional viruses, such as picorna viruses, alpha viruses, herpes viruses, parvoviruses, rhinoviruses, baculoviruses.

In another embodiment of the present invention, a vector comprising substantially all of the adenoviral genome is disclosed. In this embodiment, the regulatory elements of the adenoviral genome, such as the E1 genes, are under the regulatory control of the host cell's associated sequences. In one aspect, post-

transcriptional or post-translational host cell effects, such as the permissivity for intron excission or complex enzyme formation, mediate the control of the vector's nucleotide sequence expresson.

In one embodiment, a vector comprises a nucleotide sequence that encodes a therapeutic agent. The term "therapeutic" is used in a generic sense and includes treating agents, prophylactic agents, and replacement agents. A therapeutic agent can be considered therapeutic if it relieves (partially or completely) or prevents at least one symptom of a disease or medical condition. In one aspect, the vectors and/or methods are utilized for the treatment of cancer.

DNA sequences encoding therapeutic agents which comprise a vector of the present invention include, but are not limited to, sequences encoding tumor necrosis factor (TNF) genes, such as TNF α; encoding interferons such as Interferon-α, Interferon-β, and Interferon-γ; encoding interleukins such as IL-1, IL2, and Interleukins 3 through 18; encoding G-CSF or GM-CSF; encoding other cytokines; encoding cellular growth factors, such as lymphokines, which are growth factors for lymphocytes; encoding epidermal growth factor (EGF) or its receptor (EGF-r), and keratinocyte growth factor (KGF); encoding soluble CD4; Factor VIII; Factor IX; cytochrome b; glucocerebrosidase; T-cell receptors; the LDL receptor, ApoE, ApoC, ApoAI and other genes involved in cholesterol transport and metabolism; the α-1 antitrypsin (α-1AT) gene; the insulin gene; the hypoxanthine phosphoribosyl transferase gene; negative selective markers or "suicide" genes, such as viral thymidine kinase genes, such as the Herpes Simplex Virus thymidine kinase gene, the cytomegalovirus virus thymidine kinase gene, and the varicella-zoster virus thymidine kinase gene; Fc receptors for antigen-binding domains of antibodies, antisense sequences which inhibit viral replication, such as antisense sequences which inhibit replication of hepatitis or hepatitis non-A non-B virus; antisense *c-myb* oligonucleotides; and antioxidants such as, but not limited to, manganese superoxide dismutase (Mn-SOD), catalase, copper-zinc-superoxide dismutase (CuZn-SOD), extracellular superoxide dismutase (EC-SOD), and glutathione reductase; tissue plasminogen activator (tPA); urinary plasminogen activator (urokinase); hirudin; the phenylalanine hydroxylase gene; nitric oxide synthetase; vasoactive peptides;

angiogenic peptides; the dopamine gene; the dystrophin gene; the beta-globin gene; the alpha-globin gene; the HbA gene; protooncogenes such as the *ras, src,* and *bcl* genes; tumor suppressor genes such as p53 and Rb; the LDL receptor; the heregulin-alpha protein gene, for treating breast, ovarian, gastric and endometrial cancers; monoclonal antibodies specific to epitopes contained within the γ-chain of a T-cell antigen receptor; the multidrug resistance (MDR) gene; DNA sequences encoding ribozymes; antisense polynucleotides; genes encoding secretory peptides which act as competitive inhibitors of angiotension converting enzyme, of vascular smooth muscle calcium channels, or of adrenergic receptors, and DNA sequences encoding enzymes which break down amyloid plaques within the central nervous system. Antigeneic molecules of pathogens, including HIV, anthrax, smallpox, polio, SARS agents, other viral components, bacterial and mycological agents. It is to be understood, however, that the scope of the present invention is not to be limited to any particular therapeutic agent, one skilled in thea rt will appreciate that the list just presented is a represenative list only.

In one aspect of the present embodiment, a regulatory nucleic acid region is located in a construct to regulate gene expression. In a particular aspect, the suicide nucleic acid region is selected from the group consisting of Herpes simplex virus type 1 thymidise kinase, oxidoreductase, cytosine deaminase, thymidine kinase thymidilate kinase (Tdk::Tmk) and deoxycytidine kinase.

In another aspect, a therapeutic nucleic acid is utilized whose product (a polypeptide or RNA) is capable of circulating within the circulatory system of a host organism. That is, the therapeutic product is provided not to replace or repair a defective copy present endogenously within a cell but instead enhances or augments an organism at the cellular level, examples include EPO, an antibody, GM-CSF, growth hormones, and alike.

The nucleic acid (or transgene) which encodes the therapeutic agent can be genomic DNA, cDNA, or fragments and derivatives thereof. The nucleic acid also can be the native DNA sequence or an allelic variant thereof. The term "allelic variant" as used herein means that the allelic variant is an alternative form of the

native DNA sequence which can have a substitution, deletion, or addition of one or more nucleotides, which does not alter substantially the function of the encoded protein or polypeptide (or fragment or derivative thereof). In one aspect, the DNA sequence can further include a leader sequence or portion thereof, a secretory signal or portion thereof and/or can further include a trailer sequence or portion thereof.

The DNA sequence encoding at least one therapeutic agent is under the control of a suitable promoter. Suitable promoters include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter (e.g. Genbank Accession AF072539, nt. 1-602); the Rous Sarcoma Virus (RSV, e.g. Genbank Accession U19931, nt. 38-631) promoter; inducible promoters, such as the MMR promoter (e.g. Genbank Accession AF334668, nt. 1-3557), the metallothionein promoter (eg. Genbank Accession I02334); heat shock promoters (e.g. Genbank Accession X13229); the albumin promoter (e.g. Genbank Accession AF255302); telomerase reverse transcriptpion promoter (e.g. Genbank Accession AB018788) and the ApoAI promoter (Genbank Accession J04066, nt. 1-2068). It is to be understood, however, that the scope of the present invention is not to be limited to specific foreign genes or promoters.

The adenoviral components of the vector comprising a first nucleotide sequence (or first polynucleotide), a second nucleotide sequence (or second polynucleotide), and a nucleic acid sequence that encodes for proteins for replication and packaging of the adenoviral vector can be obtained from any adenoviral serotype, including, but not limited to, Adenovirus 2, Adenovirus 3, Adenovirus 4, Adenovirus 5, Adenovirus 6 through 41, simian, canine, feline, murine and bovine Adenoviruses.

In one aspect, the adenoviral components of the first polynucleotide are obtained or derived from Adenovirus 5, and the adenoviral components of the second polynucleotide, as well as the DNA sequences necessary for replication and packaging of the adenoviral vector, are obtained or derived from the Adenovirus 5 (ATCC No. VR-5) genome or the Adenovirus 5 E3-mutant Ad d1327 (Thimmapaya,

et al, Cell, Vol. 31, pg. 543 (1983), the entire teaching of which is incorporated herein by reference).

In another aspect, the adenoviral components are derived from a combination of adenoviral genomes, for example dl1520, is a C type adenovirus chimera containing a combination of adenovirus serotype 5 and adenovirus serotype sequences (e.g. US patent 5,677,178). In yet another aspect, the adenovirus of the first or the second polynucleotide as well as the DNA sequences necessary for replication and packaging of the adenoviral vector, are not obtained or derived from the Adenovirus 5 but from a complementing functional sequence (for example, Human papilloma virus E6 and/or E7 regions).

Cells that can be subjected to infection by adenoviral vectors of the present invention include, but are not limited to, tumor cells, primary cells, such as primary nucleated blood cells, such as leukocytes, granulocytes, monocytes, macrophages, lymphocytes (including T-lymphocytes and B-lymphocytes), totipotent stem cells, and tumor infiltrating lymphocytes (TIL cells); bone marrow cells; endothelial cells, activated endothelial cells; epithelial cells; lung cells; keratinocytes; stem cells; hepatocytes, including hepatocyte precursor cells, fibroblasts; mesenchymal cells; mesothelial cells; parenchymal cells; vascular smooth muscle cells; brain cells and other neural cells; gut enterocytes; gut stem cells; myoblasts and any tumor cells.

The infected cells are useful in the treatment of a variety of diseases including, but not limited to, liver cells, whereby the adenoviral vectors include a gene encoding a therapeutic agent employed to treat acquired infectious diseases, such as diseases resulting from viral infection. For example, infectious adenoviral vectors of the present invention can be employed to treat viral hepatitis, particularly hepatitis B or non-A non-B hepatitis. For example, an infectious adenoviral vector of the instant invention comprising a gene encoding an anti-sense gene can be employed to infect liver cells in order to inhibit viral replication. In this case, the infectious adenoviral vector, which includes a structural hepatitis gene in the reverse or opposite orientation, can be introduced into liver cells, resulting in production in the infected liver cells of an anti-sense gene capable of inactivating the hepatitis virus or its RNA

transcripts. Alternatively, the liver cells can be infected with an infectious adenoviral vector which includes a gene that encodes a protein such as interferon-α, which may confer resistance to the hepatitis virus.

In another aspect, the adenoviral vectors of the present invention can be employed to infect eukaryotic cells *in vitro*. The eukaryotic cells can be those as hereinabove described. Such eukaryotic cells can be administered to a host as part of a gene therapy procedure in amounts effective to produce a therapeutic effect in a host. Alternatively, vectors comprising a gene encoding a desired protein or therapeutic agent can be employed to infect a desired cell line *in vitro*, whereby the infected cells produce a desired protein or therapeutic agent *in vitro*.

The present invention also relates to adenoviral vectors that can be pseudotyped into capsid structures based on a variety of adenoviruses. Thus, a practitioner can use the adenoviral vectors of the present invention to generate adenoviral vectors having various capsids against which humans do not have, or rarely have, pre-existing antibodies. For example, a practitioner can generate an adenoviral vector in accordance with the present invention from a plasmid having an ITR and a packaging signal obtained from Adenovirus 5, and a helper virus which contains adenoviral components obtained from the Adenovirus 5 genome. The viral vectors generated will have an Adenovirus 5 capsid. Adenovirus 5, however, is associated with the common cold, and anti-Adenovirus 5 antibodies are found in many humans. Thus, in order to decrease the possibility of the occurrence of an immune response against the adenoviral vector, the adenoviral vectors can have nucleotide sequences from other than Adenovirus 5, such as Adenovirus 4, Adenovirus 12, or simian or bovine adenoviruses, or derivatives thereof. Thus, a practitioner can generate new adenoviral vectors having capsids that are not Adenovirus 5 derived, and therefore, such vectors are less likely to be inactivated by an immune response. Alternatively, the vectors can be transfected into an adenoviral packaging cell line that includes a helper virus including DNA encoding an altered Adenovirus 5 exon, thereby generating a new adenoviral vector having an altered Adenovirus 5 capsid. It is to be understood, however, that this embodiment is not to be limited to any specific pseudotyped adenovirus.

A skilled artisan is aware of repositories for cells and plasmids. The American Type Culture Collection (http://phage.atcc.org/searchengine/all.html) contains the cells and other biological entities utilized herein and would be aware of means to identify other cell lines which would work equally well in the methods of the present invention. The HEK 293 cells can be obtained therein with the identifier ATCC 45504, and the C3 cells can be obtained with the ATCC CRL-10741 identifier. The HepG2 cells mentioned herein are obtained with ATCC HB-8065. Many adenovirus genomes, which can be utilized in vectors of the invention, include those available from the American Type Culture Collection: adenovirus type 1 (ATCC VR-1), adenovirus type 2 (ATCC CR-846), adenovirus type 3 (ATCC VR-3 or ATCC VR-847), adenovirus type 5 (ATCC VR-5), *etc.*

In another embodiment of the present invention, genetic complementation occurs between an exogenous nucleic acid and a host cell's genome, wherein an intervening exogenous nucleotide sequence replaces an endogenous nucleotide sequence of the host cell's genome, thereby forming a functional self-splicing intron. In one aspect, the host cell is an hepatocyte. In another aspect, the endogenous nucleotide sequence is selected from the group consisting of a reporter region, *ras, myc, raf, erb, src, fms, jun, trk, ret, gsp, hst, bcl abl,* Rb, CFTR, p16, p21, p27, p53, p57, p73, C-CAM, APC, CTS-1, zac1, scFV *ras*, DCC, NF-1, NF-2, WT-1, MEN-I, MEN-II, BRCA1, VHL, MMAC1, FCC, MCC, BRCA2, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11 IL-12, IL15, IL18, GM-CSF, G-CSF, TNF, gIFN, aIFN, bIFN, thymidine kinase, cytosine deaminase, cyt-p450, CD40L, Factor VIII, Factor IX, CD40, multiple disease resistance (MDR), ornithine transcarbamylase (OTC), ICAM-1, HER2-neu, PSA, terminal transferase, caspase, NOS, VEGF, endostatin, vegostatin, FGF, FGF4, bFGF, HIS, heat shock proteins, IFN α and γ, TNF α and β, telomerase, and insulin receptor. A skilled artisan is aware these sequences and any others which can be used in the invention as described above and are readily obtainable by searching a nucleic acid sequence repository such as GenBank which is available online at http://www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html.

NUCLEIC ACID-BASED EXPRESSION SYSTEMS

1. Vectors

The term "vector" is used herein to refer to a carrier molecule by which a nucleic acid sequence can be introduced into a cell, for example plasmid, viruses, such as adenoviruses, retroviruses, herpes viruses, alphaviruses and other suitable carriers. In one embodiment, the carrier molecule is a nucleic acid or a virion. A nucleic acid sequence can be "exogenous," meaning that it is foreign to the cell into which the vector is being introduced or that the sequence is homologous to a sequence in the cell but in a position within the host cell nucleic acid in which the sequence is ordinarily not found. One of skill in the art would be well equipped to construct a vector through standard recombinant techniques, which are described in Maniatis *et al.*, 1988 and Ausubel *et al.*, 1994, the teachings of which are incorporated herein by reference.

The term "expression vector" refers to a vector containing a nucleic acid sequence coding for at least part of a gene product capable of being transcribed. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. In other cases, these sequences are not translated, for example, in the production of antisense molecules or ribozymes. Expression vectors can contain a variety of "control sequences," which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operably linked coding sequence in a particular host organism. The control can be pre-transcription, transcriptional, post-transcriptional or post-translational. Specifically, it can contain regulatory elements such as promoters, enhanzers, introns or split "targezyme" introns to regulate expression. In addition to control sequences that govern transcription and translation, vectors and expression vectors can contain nucleic acid sequences that serve other functions as well and are described *infra*.

a. Promoters and Enhancers

A "promoter" is a control sequence that is a region of a nucleic acid sequence, usually positioned toward the 5' end, at which initiation and rate of transcription are

controlled. It can contain genetic elements at which regulatory proteins and other *trans*-acting molecules can bind such as RNA polymerase as well as other transcription factors. The phrases "operatively positioned," "operatively linked," "under control," and "under transcriptional control" mean that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence to control transcriptional initiation and/or expression of that sequence. A promoter may or may not be used in conjunction with an "enhancer," which refers to a *cis*-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence.

A promoter can be one naturally associated with a gene or sequence, as may be obtained by isolating the 5′ non-coding sequences located upstream of the coding segment and/or exon. Such a promoter can be referred to as "endogenous." Similarly, an enhancer can be one naturally associated with a nucleic acid sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding nucleic acid segment under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with a nucleic acid sequence in its natural environment. A recombinant or heterologous enhancer refers also to an enhancer not normally associated with a nucleic acid sequence in its natural environment. Such promoters or enhancers can include promoters or enhancers of other genes, and promoters or enhancers isolated from any other prokaryotic, viral, or eukaryotic cell, and promoters or enhancers not "naturally occurring," *i.e.*, containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences are produced using recombinant cloning and/or nucleic acid amplification technology, including PCR, in connection with the compositions disclosed herein, see U.S. Patent 4,683,202, U.S. Patent 5,928,906, the teachings of which are incorporated herein by reference. Furthermore, it is contemplated that the control sequences that direct transcription and/or expression of sequences within non-nuclear organelles such as mitochondria, chloroplasts, and the like, can be employed as well.

In an embodiment of the present invention, a vector comprising a bidirectional promoter such as the aldehyde reductase promoter described in US Pat. No. 6,630,324 to Barski *et al.*, the teaching of which is incorporated herein by refrence, in which two gene products (RNA or polypeptide) are transcribed from the same regulatory sequence is disclosed. This permits production of two gene products in relatively equivalent stoichiometric amounts.

It is important to employ a promoter and/or enhancer that effectively directs the expression of the DNA segment in the cell type, organelle, and organism chosen for expression. Those of skill in the art are familiar with the use of promoters, enhancers, and cell type combinations for protein expression, for example, see Sambrook *et al.* (1989), the entire teaching of which is incorporated herein by reference. The promoters employed herein can be constitutive, tissue-specific, inducible, and/or useful under the appropriate conditions to direct high level expression of the introduced DNA segments. The promoter can be heterologous or endogenous.

Table 1 lists several elements/promoters that can be employed to regulate the expression of a gene. This list is not intended to be exhaustive of all the possible elements involved in the promotion of expression but, merely, to be exemplary thereof. Table 2 provides examples of inducible elements, which are regions of a nucleic acid sequence that can be activated in response to a specific stimulus. The references disclosed in the tables are incorporated herein by reference.

TABLE 1

Promoter and/or Enhancer

Promoter/Enhancer	References
Immunoglobulin Heavy Chain	Banerji *et al.*, 1983; Gilles *et al.*, 1983; Grosschedl *et al.*, 1985; Atchinson *et al.*, 1986, 1987; Imler *et al.*, 1987; Weinberger *et al.*, 1984; Kiledjian *et al.*, 1988; Porton *et al.*; 1990
Immunoglobulin Light Chain	Queen *et al.*, 1983; Picard *et al.*, 1984
T-Cell Receptor	Luria *et al.*, 1987; Winoto *et al.*, 1989; Redondo *et al.*; 1990

TABLE 1

Promoter and/or Enhancer

Promoter/Enhancer	References
HLA DQ a and/or DQ β	Sullivan *et al.*, 1987
β-Interferon	Goodbourn *et al.*, 1986; Fujita *et al.*, 1987; Goodbourn *et al.*, 1988
Interleukin-2	Greene *et al.*, 1989
Interleukin-2 Receptor	Greene *et al.*, 1989; Lin *et al.*, 1990
MHC Class II 5	Koch *et al.*, 1989
MHC Class II HLA-Dra	Sherman *et al.*, 1989
β-Actin	Kawamoto *et al.*, 1988; Ng *et al.*; 1989
Muscle Creatine Kinase (MCK)	Jaynes *et al.*, 1988; Horlick *et al.*, 1989; Johnson *et al.*, 1989
Prealbumin (Transthyretin)	Costa *et al.*, 1988
Elastase I	Omitz *et al.*, 1987
Metallothionein (MTII)	Karin *et al.*, 1987; Culotta *et al.*, 1989
Collagenase	Pinkert *et al.*, 1987; Angel *et al.*, 1987
Albumin	Pinkert *et al.*, 1987; Tronche *et al.*, 1989, 1990
α-Fetoprotein	Godbout *et al.*, 1988; Campere *et al.*, 1989
t-Globin	Bodine *et al.*, 1987; Perez-Stable *et al.*, 1990
β-Globin	Trudel *et al.*, 1987
c-fos	Cohen *et al.*, 1987
c-HA-*ras*	Triesman, 1986; Deschamps *et al.*, 1985
Insulin	Edlund *et al.*, 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsh *et al.*, 1990
α_1-Antitrypain	Latimer *et al.*, 1990
H2B (TH2B) Histone	Hwang *et al.*, 1990
Mouse and/or Type I Collagen	Ripe *et al.*, 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang *et al.*, 1989
Rat Growth Hormone	Larsen *et al.*, 1986
Human Serum Amyloid A (SAA)	Edbrooke *et al.*, 1989

TABLE 1

Promoter and/or Enhancer

Promoter/Enhancer	References
Troponin I (TN I)	Yutzey *et al.*, 1989
Platelet-Derived Growth Factor (PDGF)	Pech *et al.*, 1989
Duchenne Muscular Dystrophy	Klamut *et al.*, 1990
SV40	Banerji *et al.*, 1981; Moreau *et al.*, 1981; Sleigh *et al.*, 1985; Firak *et al.*, 1986; Herr *et al.*, 1986; Imbra *et al.*, 1986; Kadesch *et al.*, 1986; Wang *et al.*, 1986; Ondek *et al.*, 1987; Kuhl *et al.*, 1987; Schaffner *et al.*, 1988
Polyoma	Swartzendruber *et al.*, 1975; Vasseur *et al.*, 1980; Katinka *et al.*, 1980, 1981; Tyndell *et al.*, 1981; Dandolo *et al.*, 1983; de Villiers *et al.*, 1984; Hen *et al.*, 1986; Satake *et al.*, 1988; Campbell and/or Villarreal, 1988
Retroviruses	Kriegler *et al.*, 1982, 1983; Levinson *et al.*, 1982; Kriegler *et al.*, 1983, 1984a, b, 1988; Bosze *et al.*, 1986; Miksicek *et al.*, 1986; Celander *et al.*, 1987; Thiesen *et al.*, 1988; Celander *et al.*, 1988; Chol *et al.*, 1988; Reisman *et al.*, 1989
Papilloma Virus	Campo *et al.*, 1983; Lusky *et al.*, 1983; Spandidos and/or Wilkie, 1983; Spalholz *et al.*, 1985; Lusky *et al.*, 1986; Cripe *et al.*, 1987; Gloss *et al.*, 1987; Hirochika *et al.*, 1987; Stephens *et al.*, 1987; Glue *et al.*, 1988
Hepatitis B Virus	Bulla *et al.*, 1986; Jameel *et al.*, 1986; Shaul *et al.*, 1987; Spandau *et al.*, 1988; Vannice *et al.*, 1988
Human Immunodeficiency Virus	Muesing *et al.*, 1987; Hauber *et al.*, 1988; Jakobovits *et al.*, 1988; Feng *et al.*, 1988; Takebe *et al.*, 1988; Rosen *et al.*, 1988; Berkhout *et al.*, 1989; Laspia *et al.*, 1989; Sharp *et al.*, 1989; Braddock *et al.*, 1989
Cytomegalovirus (CMV)	Weber *et al.*, 1984; Boshart *et al.*, 1985; Foecking *et al.*, 1986
Gibbon Ape Leukemia Virus	Holbrook *et al.*, 1987; Quinn *et al.*, 1989

TABLE 2

Inducible Elements

Element	Inducer	References
MT II	Phorbol Ester (TFA) Heavy metals	Palmiter *et al.*, 1982; Haslinger *et al.*, 1985; Searle *et al.*, 1985; Stuart *et al.*, 1985; Imagawa *et al.*, 1987, Karin *et al.*, 1987; Angel *et al.*, 1987b; McNeall *et al.*, 1989
MMTV (mouse mammary tumor virus)	Glucocorticoids	Huang *et al.*, 1981; Lee *et al.*, 1981; Majors *et al.*, 1983; Chandler *et al.*, 1983; Lee *et al.*, 1984; Ponta *et al.*, 1985; Sakai *et al.*, 1988
β-Interferon	poly(rI)x poly(rc)	Tavernier *et al.*, 1983
Adenovirus 5 E2	ElA	Imperiale *et al.*, 1984
Collagenase	Phorbol Ester (TPA)	Angel *et al.*, 1987a
Stromelysin	Phorbol Ester (TPA)	Angel *et al.*, 1987b
SV40	Phorbol Ester (TPA)	Angel *et al.*, 1987b
Murine MX Gene	Interferon, Newcastle Disease Virus	Hug *et al.*, 1988
GRP78 Gene	A23187	Resendez *et al.*, 1988
α-2-Macroglobulin	IL-6	Kunz *et al.*, 1989
Vimentin	Serum	Rittling *et al.*, 1989
MHC Class I Gene H-2κb	Interferon	Blanar *et al.*, 1989
HSP70	ElA, SV40 Large T Antigen	Taylor *et al.*, 1989, 1990a, 1990b
Proliferin	Phorbol Ester-TPA	Mordacq *et al.*, 1989
Tumor Necrosis Factor	PMA	Hensel *et al.*, 1989
Thyroid Stimulating Hormone α Gene	Thyroid Hormone	Chatterjee *et al.*, 1989

The identity of tissue-specific promoters or elements, as well as assays to characterize their activity, is well known to those of skill in the art. Examples of such regions include the human LIMK2 gene (Nomoto *et al.* 1999, the teaching of which is

incorporated herein by reference), the somatostatin receptor 2 gene (Kraus *et al.*, 1998, the teaching of which is incorporated herein by reference), murine epididymal retinoic acid-binding gene (Lareyre *et al.*, 1999, the teaching of which is incorporated herein by reference), human CD4 (Zhao-Emonet *et al.*, 1998, the teaching of which is incorporated herein by reference), mouse α-2 (XI) collagen (Tsumaki, *et al.*, 1998, the teaching of which is incorporated herein by reference), D1A dopamine receptor gene (Lee, *et al.*, 1997, the teaching of which is incorporated herein by reference), insulin-like growth factor II (Wu *et al.*, 1997, the teaching of which is incorporated herein by reference), human platelet endothelial cell adhesion molecule-1 (Almendro *et al.*, 1996, the teaching of which is incorporated herein by reference).

b. Initiation Signals and Internal Ribosome Binding Sites

A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon or adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be "in-frame" with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous translational control signals and initiation codons can be either natural or synthetic. The efficiency of expression can be enhanced by the inclusion of appropriate transcription enhancer elements.

In certain embodiments of the invention, the use of internal ribosome entry sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988, the teaching of which is incorporated herein by reference). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991, the teaching of which is incorporated herein by reference). IRES elements can be linked to heterologous open reading frames.

Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message (see U.S. Patent 5,925,565 and 5,935,819, the teachings of which are incorporated herein by reference).

c. Multiple Cloning Sites

Vectors can include a multiple cloning site (MCS), which is a nucleic acid region that contains multiple restriction enzyme sites, any of which can be used in conjunction with standard recombinant technology to digest the vector. (See Carbonelli *et al.*, 1999, Levenson *et al.*, 1998, and Cocea, 1997, incorporated herein by reference.) "Restriction enzyme digestion" refers to catalytic cleavage of a nucleic acid molecule with an enzyme that functions only at specific locations in a nucleic acid molecule. Many of these restriction enzymes are commercially available. Use of such enzymes is widely understood by those of skill in the art. Frequently, a vector is linearized or fragmented using a restriction enzyme that cuts within the MCS to enable exogenous sequences to be ligated to the vector. "Ligation" refers to the process of forming phosphodiester bonds between two nucleic acid fragments, which may or may not be contiguous with each other. Techniques involving restriction enzymes and ligation reactions are well known to those of skill in the art of recombinant technology.

d. Splicing Sites

Most transcribed eukaryotic RNA molecules will undergo RNA splicing to remove introns from the primary transcripts. Vectors containing genomic eukaryotic sequences may require donor and/or acceptor splicing sites to ensure proper processing of the transcript for protein expression. (See Chandler *et al.*, 1997, incorporated herein by reference.) In addition, splice regions have been demonstrated to be amenable to separation such as functional domains 1 and 2 of the Tetrahymena intron 1. These intron functional domains can also be evolved so a functional RNA-

self splicing complex can be formed by use of an excisting cellular RNA. Such approach can be used for tissue directed gene expression and regulation.

e. Polyadenylation Signals

In expression, a practitioner will typically include a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and/or any such sequence may be employed. Embodiments include the SV40 polyadenylation signal and/or the bovine growth hormone polyadenylation signal, convenient and/or known to function well in various target cells. Also contemplated as an element of the expression cassette is a transcriptional termination site. These elements can serve to enhance message levels and/or to minimize read through from the cassette into other sequences.

f. Origins of Replication

In order to propagate a vector in a host cell, it can contain one or more origins of replication sites (often termed "ori"), which is a specific nucleic acid sequence at which replication is initiated.

g. Selectable and Screenable Markers

In certain embodiments of the invention, where cells contain a nucleic acid construct of the present invention, a cell can be identified *in vitro* or *in vivo* by including a marker in an expression vector. Such markers confer an identifiable change to the cell permitting easy identification of cells containing the expression vector. Generally, a selectable marker is one that confers a property that allows for selection. A positive selectable marker is one in which the presence of the marker allows for its selection, while a negative selectable marker is one in which its presence prevents its selection. An example of a positive selectable marker is a drug resistance marker.

Usually the inclusion of a drug selection marker aids in the cloning and identification of transformants, for example, genes that confer resistance to neomycin,

puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selectable markers. In addition to markers conferring a phenotype that allows for the discrimination of transformants based on the implementation of conditions, other types of markers including screenable markers such as GFP, whose basis is colorimetric analysis are also contemplated. Alternatively, screenable enzymes such as herpes simplex virus thymidine kinase (*tk*) or chloramphenicol acetyltransferase (CAT) can be utilized. One of skill in the art would also know how to employ immunologic markers, possibly in conjunction with FACS analysis. The marker used is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable and screenable markers are well known to one of skill in the art.

2. Host Cells

As used herein, the terms "cell," "cell line," and "cell culture" can be used interchangeably. All of these terms also include their progeny, which are any and all subsequent generations. It is understood that all progeny may not be identical due to deliberate or inadvertent mutations. In the context of expressing a heterologous nucleic acid sequence, "host cell" refers to a prokaryotic or eukaryotic cell, and it includes any transformable organism that is capable of replicating a vector and/or expressing a heterologous gene encoded by a vector. A host cell can, and has been, used as a recipient for vectors. A host cell can be "transfected" or "transduced," which refers to a process by which exogenous nucleic acid is transferred or introduced into the host cell. A transduced cell includes the primary subject cell and its progeny.

Host cells can be derived from prokaryotes or eukaryotes, depending upon whether the desired result is construction, replication of the vector or expression of part or all of the vector-encoded nucleic acid sequences. Numerous cell lines and cultures are available for use as a host cell, and they can be obtained through the American Type Culture Collection (ATCC), which is an organization that serves as an archive for living cultures and genetic materials (www.atcc.org). An appropriate host can be determined by one of skill in the art based on the vector backbone and the desired result. A plasmid or cosmid, for example, can be introduced into a prokaryote

host cell for replication of many vectors. Bacterial cells used as host cells for vector replication and/or expression include DH5α, JM109, and KC8, as well as a number of commercially available bacterial hosts such as SURE® Competent Cells and SOLOPACK™ Gold Cells (STRATAGENE®, La Jolla). Alternatively, bacterial cells such as *E. coli* LE392 could be used as host cells for phage viruses.

Examples of eukaryotic host cells for replication and/or expression of a vector include HeLa, NIH3T3, Jurkat, 293, Cos, CHO, Saos, and PC12. Many host cells from various cell types and organisms are available and are known to one of skill in the art. Similarly, viral vectors can be used in conjunction with either eukaryotic or prokaryotic host cells, particularly those that are permissive for replication or expression of the vector.

Some vectors can employ control sequences that allow it to be replicated and/or expressed in both prokaryotic and eukaryotic cells. One of skill in the art understands the conditions under which to incubate all of the above described host cells to maintain them and to permit replication of a vector. Also understood and known are techniques and conditions that would allow large-scale production of vectors, as well as production of the nucleic acids encoded by vectors and their cognate polypeptides, proteins, or peptides (in Methods in molecular Biology. Gene transfer and expression protocols (Murray EJ, ed.) Clifton, NJ: The Humana Press, Inc., incorporated by reference; and e.g. Mulligan, R, 1993; Faustinella, et al 1994; Haddada, et al 1995; Smith AE, 1995; Ali, et al 1995; Bilbao et al 1997; Miyake, et al 1997; Morgan and Anderson, 1993).

3. Expression Systems

Numerous expression systems exist that comprise at least a part or all of the compositions discussed above. Prokaryote- and/or eukaryote-based systems can be employed for use with the present invention to produce nucleic acid sequences, or their cognate polypeptides, proteins and peptides. Many such systems are commercially and widely available.

The insect cell/baculovirus system can produce a high level of protein expression of a heterologous nucleic acid segment, such as described in U.S. Patent Nos. 5,871,986 & 4,879,236, both of which are incorporated herein by reference, and which can be obtained, for example, under the tradenames MAXBAC® 2.0 from INVITROGEN® and BACPACK™ BACULOVIRUS EXPRESSION SYSTEM FROM CLONTECH®.

Other examples of expression systems include STRATAGENE®'s COMPLETE CONTROL™ Inducible Mammalian Expression System, which involves a synthetic ecdysone-inducible receptor, or its pET Expression System, an *E. coli* expression system. Another example of an inducible expression system is available from INVITROGEN®, which carries the T-REX™ (tetracycline-regulated expression) System, an inducible mammalian expression system that uses the full-length CMV promoter. INVITROGEN® also provides a yeast expression system called the *Pichia methanolica* Expression System, which is designed for high-level production of recombinant proteins in the methylotrophic yeast *Pichia methanolica*. One of skill in the art would know how to express a vector, such as an expression construct, to produce a nucleic acid sequence or its cognate polypeptide, protein, or peptide.

2. Gene Therapy Administration

For gene therapy, a skilled artisan understands that a vector to be utilized must contain sequences or genes of interest operatively linked to promoters (Mulligan, 1993). For antisense gene therapy, the antisense sequence of the gene of interest would be operatively linked to a promoter. One skilled in the art recognizes that in certain instances other sequences such as a 3' UTR regulatory sequences are useful in expressing the gene of interest. Where appropriate, the gene therapy vectors can be formulated into preparations of solid, semisolid, liquid or gaseous forms in the ways known in the art for their respective route of administration. Means known in the art can be utilized to prevent release and absorption of the composition until it reaches the target organ or to ensure timed-release of the composition. A pharmaceutically acceptable form should be employed which does not attenuate the desired properties of the compositions of the present invention. In pharmaceutical dosage forms, the

compositions can be used alone or in appropriate association, as well as in combination, with other pharmaceutically active compounds. An effective amount of vector containing the therapeutic nucleic acid sequence is administered to provide a pharmacologically effective dose of the gene product. An effective amount can be understood as that amount necessary to effectuate an observable change (*e.g.*, a therapeutic effect) and can be determined without undue experimentation.

One skilled in the art recognizes that different methods of delivery can be utilized to administer a vector into a cell, examples of which include: (1) methods utilizing physical means, such as electroporation (electricity), a gene gun (physical force) or applying large volumes of a liquid (pressure); (2) methods wherein said vector is complexed to another entity, such as a liposome or transporter molecule; and methods wherein said vector contains a protein factor that attaches and gets incorporated into the cell, such as occurs with virions.

Accordingly, the present invention provides a method of transferring a therapeutic gene to a host, which comprises administering the vectors of the present invention using any of the aforementioned routes of administration or alternative routes known to those skilled in the art and appropriate for a particular application. Effective gene transfer of the vectors to a host cell in accordance with the present invention can be monitored in terms of a therapeutic effect (*e.g.*, alleviation of some symptom associated with the particular disease being treated) or, further, by evidence of the transferred gene or expression of the gene within the host (*e.g.*, using the polymerase chain reaction in conjunction with sequencing, Northern or Southern hybridizations, or transcription assays to detect the nucleic acid in host cells, or using immunoblot analysis, antibody-mediated detection, mRNA or protein half-life studies, or particularized assays to detect protein or polypeptide encoded by the transferred nucleic acid, or impacted in level or function due to such transfer).

The methods described herein are by no means all-inclusive, and further methods to suit the specific application will be apparent to the ordinary skilled artisan. Moreover, the effective amount of the compositions can be further approximated through analogy to compounds known to exert the desired effect.

Furthermore, the actual dose and schedule can vary depending on whether the compositions are administered in combination with other pharmaceutical compositions, or depending on inter-individual differences in pharmacokinetics, drug disposition, and metabolism. Similarly, amounts can vary in *in vitro* applications depending on the particular cell line utilized (*e.g.*, based on the number of vector receptors present on the cell surface, or the ability of the particular vector employed for gene transfer to replicate in that cell line). Furthermore, the amount of vector to be added per cell can vary with the length and stability of the therapeutic gene inserted in the vector, as well as the nature of the sequence, and is a parameter which needs to be determined empirically, and can be altered due to factors not inherent to the methods of the present invention (for instance, the cost associated with synthesis). One skilled in the art can easily make any necessary adjustments in accordance with the exigencies of the particular situation.

It is possible that cells containing the therapeutic gene can also contain a suicide gene (*i.e.*, a gene which encodes a product that can be used to destroy the cell, such as herpes simplex virus thymidine kinase) as a therapeutic or safety gene. As a therapeutic gene, for example in the treatment of cancer, the suicide gene's cytotoxic effects are the desired outcome. In many gene therapy situations, it is desirable to be able to express a gene for therapeutic purposes in a host cell but also to have the capacity to destroy the host cell once the therapy is completed. This goal can be accomplished using the present invention by having one nucleotide sequence being the therapeutic gene linked to a promoter and having a second nucleotide sequence being the suicide gene also linked to the promoter. Thus, expression of the therapeutic gene in a host cell can be driven by a pre-determined promoter, although the product of a suicide gene will typically remain harmless in the absence of a prodrug. Once the therapy is complete, or no longer desired or needed, administration of a prodrug causes the suicide gene product to turn lethal to the cell. Examples of suicide gene/prodrug combinations that can be used are Herpes Simplex Virus-thymidine kinase (HSV-tk) and ganciclovir, acyclovir or FIAU; oxidoreductase and cycloheximide; cytosine deaminase and 5-fluorocytosine; thymidine kinase thymidilate kinase (Tdk::Tmk) and AZT; and deoxycytidine kinase and cytosine arabinoside.

The method of cell therapy can be employed by methods known in the art, wherein a cultured cell containing a copy of a nucleic acid sequence or amino acid sequence of a sequence of interest is introduced using, for example, methods discussed above such as electroporation.

3. Combination Treatments

In one embodiment of the present invention, the vectors and methods using the vectors described herein employ a nucleic acid that is therapeutic for the treatment of cancer. In order to increase the effectiveness of gene therapy with an anti-cancer nucleic acid sequence of interest, it may be desirable to combine these compositions with other agents effective in the treatment of hyperproliferative disease, such as anti-cancer agents. An "anti-cancer" agent is capable of negatively affecting cancer in a subject, for example, by killing cancer cells, inducing apoptosis in cancer cells, reducing the growth rate of cancer cells, reducing the incidence or number of metastases, reducing tumor size, inhibiting tumor growth, reducing the blood supply to a tumor or cancer cells, promoting an immune response against cancer cells or a tumor, preventing or inhibiting the progression of cancer, or increasing the lifespan of a subject with cancer. More generally, these other compositions can be provided in a combined amount effective to kill or inhibit proliferation of an affected cell. This process can involve contacting the cell with an expression construct and the agent(s) or multiple factor(s) at the same or near the same time. This can be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell with at least two distinct compositions or formulations, at the same time, wherein one composition includes the expression construct and the other includes the second agent(s).

Tumor cell resistance to chemotherapy and radiotherapy agents represents a major concern in clinical oncology. One goal of current cancer research is to find ways to improve the efficacy of chemo- and radiotherapy by combining it with gene therapy. For example, the herpes simplex-thymidine kinase (HSV-tk) gene, when delivered to brain tumors by a retroviral vector system, successfully induced susceptibility to the antiviral agent ganciclovir (Culver, *et al.*, 1992, the entire teaching of which is incorporated herein by reference). In the present invention, it is

contemplated that HSV-tk gene therapy, in the context of adenoviral or other viral backbones, could be used similarly in conjunction with chemotherapeutic, radiotherapeutic, or immunotherapeutic intervention, in addition to other pro-apoptotic or cell cycle regulating agents.

Alternatively, gene therapy can precede or follow other agent treatment by intervals ranging from minutes to weeks. In embodiments where another agent and expression construct are applied separately to the cell, a practitioner would generally ensure that a significant period of time did not expire between the time of each delivery, such that the agent and expression construct would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one can contact the cell with both modalities within about 12-24 h of each other and, more preferably, within about 6-12 h of each other. In some situations, it may be desirable to extend the time period for treatment significantly where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

Various combinations can be employed, gene therapy is "A" and the secondary agent, such as radio- or chemotherapy, is "B":

A/B/A	B/A/B	B/B/A	A/A/B	A/B/B	B/A/A	A/B/B/B	B/A/B/B
B/B/B/A	B/B/A/B	A/A/B/B	A/B/A/B	A/B/B/A	B/B/A/A		
B/A/B/A	B/A/A/B	A/A/A/B	B/A/A/A	A/B/A/A	A/A/B/A		

Administration of a therapeutic expression construct of the present invention to a subject follows general protocols for the administration of chemotherapeutics, taking into account the toxicity, if any, of the vector. Treatment cycles may require to be to repeated. Also, is contemplated that various standard therapies, as well as surgical intervention, can be applied in combination with the described hyperproliferative cell therapy.

a. Chemotherapy

Cancer therapies also include a variety of combination therapies with both chemical and radiation based treatments. Combination chemotherapies include, for example, cisplatin (CDDP), carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, busulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicomycin, mitomycin, etoposide (VP16), tamoxifen, raloxifene, estrogen receptor binding agents, taxol, gemcitabien, navelbine, farnesyl-protein tansferase inhibitors, transplatinum, 5-fluorouracil, vincristin, vinblastin and methotrexate, or any analog or derivative variant of the foregoing.

In another form, chemotherapies can be composed of proteins such as hormones, antibodies, antiangiogenic factors, molecular traps or other chemical compositions known to someone skilled in the art.

b. Radiotherapy

Other factors that cause DNA damage and have been used extensively include what are commonly known as g-rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves and UV-irradiation. It is most likely that all of these factors effect a broad range of damage on DNA, on the precursors of DNA, on the replication and repair of DNA, and on the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 wk), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

The terms "contacted" and "exposed," when applied to a cell, are used herein to describe the process by which a therapeutic construct and a chemotherapeutic or radiotherapeutic agent are delivered to a target cell or are placed in direct

juxtaposition with the target cell. To achieve cell killing or stasis, both agents are delivered to a cell in a combined effective amount to kill the cell or prevent it from dividing.

c. Immunotherapy

Immunotherapeutics, generally, rely on the use of immune effector cells and molecules to target and destroy cancer cells. The immune effector can be, for example, an antibody specific for some marker on the surface of a tumor cell. The antibody alone can serve as an effector of therapy or it can recruit other cells to actually effect cell killing. The antibody can also be conjugated to a drug or toxin (chemotherapeutic, radionuclide, ricin A chain, cholera toxin, pertussis toxin, *etc.*) and serve merely as a targeting agent. Alternatively, the effector can be a lymphocyte carrying a surface molecule that interacts, either directly or indirectly, with a tumor cell target. Various effector cells include cytotoxic T cells and NK cells.

Immunotherapy, thus, could be used as part of a combined therapy, in conjunction with mixed vectors gene therapy. The general approach for combined therapy is discussed below. Generally, the tumor cell must bear some marker that is amenable to targeting, *i.e.*, is not present on the majority of other cells. Many tumor markers exist and any of these may be suitable for targeting in the context of the present invention. Common tumor markers include carcinoembryonic antigen, prostate specific antigen, urinary tumor associated antigen, fetal antigen, tyrosinase (p97), gp68, TAG-72, HMFG, Sialyl Lewis Antigen, MucA, MucB, PLAP, estrogen receptor, EGF-receptor, laminin receptor, *erb* B and p155.

d. Genes

In yet another embodiment, secondary treatment includes additional gene therapy in which additional therapeutic polynucleotides are administered before, after, or at the same time as the combined therapeutic polynucleotides comprising all or part of a nucleic acid sequence of interest. Delivery of a vector encoding either a full length or truncated amino acid sequence of interest in conjuction with an additional vector encoding one of the following gene products will have a combined anti-

hyperproliferative effect on target tissues. Alternatively, a single vector encoding multiple genes can be used.

e. Surgery

Approximately 60% of persons with cancer will undergo surgery of some type, which includes preventative, diagnostic or staging, curative and palliative surgery. Curative surgery is a cancer treatment that can be used in conjunction with other therapies, such as the treatment of the present invention, chemotherapy, radiotherapy, hormonal therapy, gene therapy, immunotherapy and/or alternative therapies.

Curative surgery includes resection in which all or part of cancerous tissue is physically removed, excised, and/or destroyed. Tumor resection refers to physical removal of at least part of a tumor. In addition to tumor resection, treatment by surgery includes laser surgery, cryosurgery, electrosurgery, and miscopically controlled surgery (Mohs' surgery). It is further contemplated that the present invention can be used in conjunction with removal of superficial cancers, precancers, or incidental amounts of normal tissue.

Gene transfer can occur before, during, after surgery or in any combination of these times. Before surgery, the delivery can be to a tumor site. Upon excision of part or all of cancerous cells, tissue, or tumor, a cavity can be formed in the body. Treatment can be effectuated by perfusion, direct injection or local application of the area with an additional anti-cancer therapy. Such treatments can be repeated, for example, every 1, 2, 3, 4, 5, 6, or 7 days, or every 1, 2, 3, 4, and 5 weeks or every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months. These treatments can be of varying dosages as well.

f. Other agents

It is contemplated that other agents can be used in combination with the present invention to improve the therapeutic efficacy of treatment. These additional agents include immunomodulatory agents, agents that affect the upregulation of cell

surface receptors and GAP junctions, cytostatic and differentiation agents, inhibitors of cell adehesion, or agents that increase the sensitivity of the hyperproliferative cells to apoptotic inducers. Immunomodulatory agents include tumor necrosis factor; interferon α, β, and γ; IL-2 and other cytokines; F42K and other cytokine analogs; or MIP-1, MIP-1β, MCP-1, RANTES, and other chemokines. It is further contemplated that the upregulation of cell surface receptors or their ligands such as Fas/Fas ligand, DR4 or DR5/TRAIL can potentiate the apoptotic inducing abililties of the present invention by establishment of an autocrine or paracrine effect on hyperproliferative cells. Increases intercellular signaling by elevating the number of GAP junctions would increase the anti-hyperproliferative effects on the neighboring hyperproliferative cell population. In other embodiments, cytostatic or differentiation agents can be used in combination with the present invention to improve the anti-hyerproliferative efficacy of the treatments. Inhibitors of cell adehesion are contemplated to improve the efficacy of the present invention. Examples of cell adhesion inhibitors are focal adhesion kinase (FAKs) inhibitors and Lovastatin. It is further contemplated that other agents that increase the sensitivity of a hyperproliferative cell to apoptosis, such as the antibody c225, could be used in combination with the present invention to improve the treatment efficacy.

Hormonal therapy can also be used in conjunction with the present invention or in combination with any other cancer therapy previously described. The use of hormones can be employed in the treatment of certain cancers such as breast, prostate, ovarian, or cervical cancer to lower the level or block the effects of certain hormones such as testosterone or estrogen. This treatment is often used in combination with at least one other cancer therapy as a treatment option or to reduce the risk of metastases.

EXAMPLES

The present invention encompasses a chimeric nucleic acid vector comprising adenoviral inverted terminal repeat flanking sequences; an internal sequence between said adenoviral flanking sequences, wherein said internal sequence contains an Rous

Sarcoma Virus (RSV) promoter driving the expression of a HSV-tk gene and most, but not all, of the viral genome sequences leading to a replication deficient (RD) adenoviral genome (Genback Accession BK000408).

A second vector comprises adenoviral inverted terminal repeat flanking sequences with a mutated adenoviral genome comprising mutations in the E1B region of the wild type virus to yield a replication conditional (RC) oncolytic vector (*e.g.* dl1520, US patent 5,677,178).

The RD vector of the present invention encodes other or additional genes such as Interleukin-2 (US patent 4,738,927), GM-CSF (Genback Accession NM_000758), or others.

The RC vector of the present invention encodes all the viral genes with promoters for tissue or condition specific replication, such as prostate specific antigen promoter (Genback Accession S81389) or the E2F promoter (Genback Accession U92807).

This system can be particularly useful for amplifying the therapeutic effects of the therapeutic gene or genes (*e.g.* HSV-tk) as well as amplifying the oncolytic effect of the RC vector (*e.g.* dl1520).

EXAMPLE 1

An example to quantitate therapeutic gene amplification can be established by injecting model tumors with a marker RD vector and a RC vector. Tumor cells, such as the human lung tumor line A549, can be injected in the flanks of immunodeficient mice. Human cells are more permissive than mouse cells to human adenovirus replication. Ten to thirty days later the tumors can be injected with 1e8 to 1e11 vp of each vector in a mixture of 10:1 to 1:10 RC:RD in 10 μL to 40 μL. To simplify pharmacokinetic measurements, a secreated marker gene, such as human growth hormone or secreted alkaline phosphatase can be used. An empty RD virion can be used to maintain a constant viral load. Six groups can be established with at least five animals per group: (1) an empty virus control (AdV-e); (2) a RD only group AdV-m + AdV-e; (3) a RC only group dl1520 + AdV-e; (4) a 10:1 RD and RC mixed group

AdV-m + dl1520; (5) a 1:1 RD and RC mixed group AdV-m + dl1520; (6) a 1:10 RD and RC mixed group AdV-m + dl1520. The mice can be bled once every two days starting 24hrs after vector injection and the serum content of the marker gene is measured.

EXAMPLE 2

An example to demonstrate benefit from therapeutic gene amplification can be established by injecting model tumors. Tumor cells, such as the human lung tumor line A549, can be injected in the flanks of immunodeficient mice. Human cells are more permissive than mouse cells to human adenovirus replication. Seven groups would be established with at least five animals per group: (1) no-virus control; (2) an empty virus control (AdV-e); (3) a RD only group AdV-tk + AdV-e; (4) a RC only group dl1520 + AdV-e; (5) a 10:1 RD and RC mixed group AdV-tk + dl1520; (6) a 1:1 RD and RC mixed group AdV-tk + dl1520; (7) a 1:10 RD and RC mixed group AdV-tk + dl1520.

EXAMPLE 3

An example to quantitate therapeutic gene amplification in the context of an imunne competent system can be established by injecting syngeneic model tumors. Tumor cells, such as the C57bl RM1 prostate tumor cell line, would be injected in the flanks of C57bl mice. These cells are less permissive than human cells but still support some human adenovirus replication. Seven to fourteen days later the tumors would be injected with 1e8 to 1e11 vp of each vector in a mixture of 10:1 to 1:10 RC:RD in 10 μL to 40 μL. To simplify pharmacokinetic measurements a secreated marker gene, such as human growth hormone or secreted alkaline phosphatase, can be used. An empty RD virion can be used to maintain a constant viral load. Six groups can be established with at least five animals per group: (1) an empty virus control (AdV-e); (2) a RD only group AdV-m + AdV-e; (3) a RC only group dl1520 + AdV-e; (4) a 10:1 RD and RC mixed group AdV-m + dl1520; (5) a 1:1 RD and RC mixed group AdV-m + dl1520; (6) a 1:10 RD and RC mixed group AdV-m + dl1520. The mice are bled once every two days starting 24hrs after vector injection and the serum content of the marker gene is measured.

EXAMPLE 4

An example to demonstrate benefit from therapeutic gene amplification in immunocompetent mice can be established by injecting syngeneic model tumors. Tumor cells, such as the C57bl RM1 prostate tumor cell line, would be injected in the flanks of C57bl mice. These cells are less permissive than human cells but still support some human adenovirus replication. Seven groups can be established with at least five animals per group: (1) no-virus control; (2) an empty virus control (AdV-e); (3) a RD only group AdV-tk + AdV-e; (4) a RC only group dl1520 + AdV-e; (5) a 10:1 RD and RC mixed group AdV-tk + dl1520; (6) a 1:1 RD and RC mixed group AdV-tk + dl1520; (7) a 1:10 RD and RC mixed group AdV-tk + dl1520.

Cells and Media

The cells can be grown and maintained in GVL (Hyclone, Logan, UT) media.

Virus

HEK293 cells can be used for adenoviral vector amplification. Transfection can be performed according to the protocol described by Cullen,[31] the teaching of which is incorporated herein by reference. Cells are left in these solutions for 10-14 hours, after which the infection/transfection media can be replaced with 20 mL fresh GVL. Approximately 30 hours post-transfection, cells can be harvested, suspended in l0 mM Tris-C1 (pH 8.0) buffer (0.5 ml/150 cm^2 plate), and frozen at –80° C. The frozen cell suspensions can be lysed by three sequential freeze (ethanol-dry ice)-thaws (37° C) cycles. Cell debris can be removed by centrifugation (3000 g for 10 minutes). Clarified extracts can be layered onto a CsC1 step gradient composed of three 5.0 mL tiers with densities of 1.45, 1.36, and 1.20 g/ml CsC1 in Tris-C1 (pH 8.0) buffer. Centrifugations can be performed at 20,000 rpm in a Beckman SW-28 rotor for 2 hours at 10°C. Fractions with visible vector bands can be collected and dialyzed against 20 mM Tris (pH 8.0), 2 mM $MgC1_2$, and 4% sucrose, then stored at –80°C in the presence of 10% glycerol.

Vertebrate Animals

Immunodeficient nude or scid mice can be used. Also, C57B1/6^J mice can be used because these animals have been used in numerous liver directed gene therapy studies.

Description of the use of animals

Approximately 100 C57Bl/6j mice, obtained from Jackson Laboratories, can be used *for in vivo* experiments. With food and water available *ad libitum*, the animals can be housed and maintained on a 12-hour light/dark cycle. All studies can be conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The mixed vectors of the present invention can be used for the delivery and stable integration of therapeutic constructs. This mixed system can eliminate some of the limitations currently encountered with *in vivo* applications of available gene transfer systems. Viral mediated gene transfer studies can be ideally performed *in vivo*. *In vivo* liver studies require animals as the source of the tissue preparations. Further, studies on viral pathogenesis can only be performed *in situ* where diverse interrelated factors that affect virulence, such as viral mutants, natural host resistance, and immunity coexist. Tissue culture systems and computer models do not reflect the complexities that occur *in vivo*.

Animal care, husbandry, and experimental factors

Mice can be anesthetized by an intraperitoneal (i.p.) injection of 0.02 mL/gm of Avertin (1.25% tribromoethanol/amyl alcohol solution). Tumor or tail vein infusion of vector solutions can be performed via a 27- or 30-gauge catheter over an approximate 5-10 minute period. These procedures are well tolerated and produce no discomfort. Tissues can be removed after euthanasia.

Euthanasia

All animals are euthanized by a 1 mL lethal injection of sodium nembutal delivered intraperitoneally.

One skilled in the art readily appreciates that the present invention is well adapted to carry out the objectives and obtain the ends and advantages mentioned as well as those inherent therein. Sequences, methods, vectors, plasmids, complexes, compounds, mutations, treatments, pharmaceutical compositions, compounds, kits, procedures and techniques described herein are presently representative of the preferred embodiments and are intended to be exemplary and are not intended as limitations of the scope. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention.

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ABSTRACT

Replication able viral vector sequences mixed with replication deficient viral vector(s) delivered *in vivo* to vector producing cells is disclosed herein. The replication able viral vector has a target cell-activated regulatory sequence or a functional mutation that controls its propagation in the target cells. Alternatively, the vectors can be mutually dependent for replication and physiological effect. The replication deficient vector(s) contains a gene encoding a protein that is expressed in the transduced cells and can induce anticancer responses. The target cells can be carcinomas, sarcomas or other solid tumors, for example. The vectors of this invention can also be utilized to treat other diseases such as restenosis, in which case the target cell can be a vascular smooth muscle cell.

APPLICATION DATA SHEET (ADS)

Application Information

Application Type::	Provisional
Subject Matter::	Utility
Title::	MIXED COMPLEMENTARY VIRAL VECTORS FOR GENE THERAPY
Attorney Docket Number::	13087-106
Suggested Drawing Figure::	0
Total Drawing Sheets::	0
Small Entity::	YES

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For ign Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::
This Application			

Assignee Information

Assignee Name::